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David J. Puri 9/00/97  
PI - Signature Date

**Lori Pierce, M.D.**

**The Effect of Radiotherapy Upon Primary Human Mammary Epithelial Cells  
Which Harbor a Breast Cancer Susceptibility Gene**

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The Effect of Radiotherapy Upon Primary Human Mammary Epithelial Cells  
Which Harbor a Breast Cancer Susceptibility Gene

**Introduction**

Inherited breast cancers account for 5 to 10% of all breast malignancies, and cancers associated with mutations in BRCA-1 and BRCA-2 are estimated to account for approximately 45% of the cases of hereditary breast cancer. The functions of the BRCA-1 and -2 genes are unknown. Recent data, however, suggest that both may be involved in DNA repair (1,2). Using an embryonic expression pattern of the mouse BRCA-2 gene, a direct interaction between the BRCA-2 protein and the DNA-repair protein MmRad 51 (the mouse homologue of Rad 51) has now been identified (1). This suggests that BRCA-2 is an essential cofactor in the Rad-51-dependent DNA repair of double-strand breaks. Studies by Sharan et al. also demonstrated that embryonic mouse cells in which the murine version of BRCA-2 had been inactivated could not recover from gamma radiation damage (1). Colocalization and coimmunoprecipitation studies have also shown that the BRCA-1 protein interacts indirectly with HsRad 51, a human homolog of bacterial RecA (2). This suggestion of a functional interaction between the two proteins may mean that BRCA-1 is involved in the maintenance of genomic integrity and perhaps DNA repair mechanisms of the cell.

Concerns have therefore been raised as to whether these cancer-predisposing genes have an enhanced radiosensitivity compared to breast cells without a breast cancer susceptibility gene mutation. Therefore, this grant will examine whether there is an increased susceptibility to radiation-induced transformation in mammary epithelial cells which either are known to have a BRCA-1 or 2 mutation or are from patients at high risk for having a mutation, and compare the results with those obtained from cells from patients not at risk for carrying a mutated breast cancer susceptibility gene.

The goal of this work is to determine whether breast cancer predisposition is correlated with enhanced cellular sensitivity to radiotherapy. Enhanced sensitivity to radiation damage will be assessed by determining whether immortalized irradiated cells from high risk patients are more likely to exhibit growth factor independence and anchorage-independent growth compared to cells without a breast cancer susceptibility gene, and to observe for any differences in rates of tumorigenicity *in vivo* following radiotherapy compared to irradiated cells that do not carry a germline mutation.

## Body

The initial aims of this grant are to determine whether cells from patients at high risk for harboring a germline mutation for a breast cancer susceptibility gene are more susceptible to immortalization following therapeutic doses of radiation *in vitro* compared to cells from reduction mammoplasty specimens from patients not at risk for hereditary breast cancer, and to determine whether immortalized irradiated cells from patients at risk for a germline mutation were more likely to exhibit growth factor independence and anchorage-independent growth relative to immortalized cells from patients not at risk for carrying a breast cancer susceptibility gene.

As designated in the grant, cell lines 6-16, 7-31, and 4-12 have been taken from patients at high risk for carrying either a BRCA-1 or 2 mutation. Although 4-12 was shown, using single stranded conformation polymorphism (SSCP) testing, to have a mutation at exon 24 of the BRCA-1 gene, subsequent sequencing of this mutation did not reveal a known functional mutation, i.e., was not associated with a truncated protein. Therefore, the genetic alteration detected via SSCP has been conservatively assumed to be a polymorphism. Additional cell lines have been established over the past year from women, who by family history, are at high risk for carrying either a BRCA-1 or 2 mutation. These are denoted as 11-21, 5-24, and SUM 194. The results of experiments with these cells are compared with parallel experiments done with cell lines from patients with no family history of female or male breast cancer or ovarian cancer (i.e., at minimal risk for carrying a BRCA-1 or 2 mutation). These control cell lines are named 1-30, 1-24, and 11-6.

We have spent considerable time analyzing the cell lines from the high risk patients in attempt to isolate a functional BRCA-1 mutation. SSCP and, more recently, conformation gel electrophoresis (CGE) have been performed on 4-12, 7-31, 6-16, and 4-12 using primers from the following exons: 2, 5, 13, 15, 16, 20, 21, 24, and 11 (a,b,c,d,e,f,g,h,i2). A genetic alteration was detected via both SSCP and CGE using primer 11 i 2 in cell lines 11-6, 7-31, and 5-24 (Figures 1 and 2). This was sequenced and found to be a known polymorphism. Now cell lines 6-16, 7-31, 11-21, and 4-12 are currently being tested for a mutation along the entire length of the BRCA-1 gene using sequential conformation gel electrophoresis.

While testing for the presence of a mutation is ongoing, we have conducted the baseline experiments, as designated in the Statement of Work (SOW). We observed both irradiated and non-irradiated high risk and control cell lines in culture for 3 months for spontaneous immortalization, as evidenced by development of clonal population of cells. The irradiation schema used was 2 Gy fractions daily, 5 fractions weekly, to a total of 30 Gy. This fractionation was chosen since it was previously

associated with the malignant transformation of a mammary cell line by Wazer et. al (3). We did not observe any evidence of spontaneous immortalization of either the high-risk or control mammary epithelial cell lines.

Next, the doubling times of the non-irradiated mammary epithelial cell lines were determined. This was done with the intent to establish a baseline for comparison with irradiated cell lines. The doubling times varied significantly by passage number within each cell line, quantitating progression to senescence. For example, the doubling time for cell line 7-31 passage (P)1 was 52.9 hours, P2 77.6 hours, and P3 136.6 hours; corresponding doubling times for 6-16 were 52.3, 64.8, and 178.4 hours, respectively; and 66.5, 70.3, and 123.8 hours for cell line 11-21. Passage 1 cells were not available for control line 1-24; however, P2 and P3 times were 66.9 and 95.9 hours, respectively. Because doubling time varied significantly with passage number of the cells, cells proceeded to senescence after 4-6 passages in culture, and we had few vials of cells at low passage numbers, we were not able to use immortalization of mammary epithelial cells as an endpoint for comparison of irradiated high risk versus control cell lines, as planned in Aim 1 of the grant. All subsequent experiments were done with cells immortalized using infection with the E6 and E7 genes from the human papilloma 16 virus. Doubling times for cell lines 1-30 HPV passage # 7, 6-16 HPV passage # 18, and 4-12 HPV passage # 24 were similar, as expected, at 42 hours, 55.8 hour, and 46.6 hours, respectively.

Because it has been shown that human breast cancer cells express cytokeratin markers consistent with an origin from the luminal cells of the terminal ductal lobular unit, immunocytochemical analysis with antibodies specific for the luminal marker keratin-19 (K-19) was performed on cell lines 1-30 (HPV), 4-12, 6-16, 7-31, 11-21, 11-6, and 1-24 to insure that cells of the appropriate lineage for malignant transformation were present in culture. All cell lines were K-19 positive with the exception of 6-16 and 7-31. These cell lines were, however, positive on staining with pankeratin antibodies. We are currently restaining these 2 cell lines with the K18 antibody, also indicative of luminal cells, and are staining the newly acquired cell lines 5-24 and SUM 194 for K-19.

Since growth factor independence has long been recognized to play an important role in tumor development and progression, one of the aims of the grant is to compare growth factor independence in irradiated high risk cell lines to control cell lines as an indication of increased tumorigenicity. In preparation for these experiments, the growth factor requirements of the unirradiated HPV cell lines had to be established. All cells were grown in serum-supplemented media 5% IHEC (Ham's F-12 medium supplemented with insulin, hydrocortisone, epidermal growth

factor (EGF), cholera toxin, and 5% fetal calf serum). The growth requirements of 4-12, 6-16, 7-31, 1-30, and 11-6 (HPV) were tested by plating cells in the following serum depleted medias: SFIHEC (serum-free media supplemented with insulin, hydrocortisone, epidermal growth factor, and cholera toxin), SFIHC (SFIHEC minus epidermal growth factor), SFHEC (SFIHEC minus insulin), SFIHE (minus cholera toxin), and SFIH (minus epidermal growth factor, cholera toxin). Cell counts in each of the serum-depleted medias were comparable to those in 5% IHEC in the first passage (Figure 3). We next looked at the growth requirements in passage 2 to determine the requirement for EGF. Cells were grown in SFIHC and each cell line demonstrated increasing cell death with increasing days in culture (Figure 4). Therefore, infection with E6 and E7 did not confer EGF independence. Thus, development of EGF independence would be a marker of phenotypic change should it be observed in the irradiated high risk cells. Experiments in which the other growth factors are deleted in passage 2 (i.e., insulin, hydrocortisone, and cholera toxin) are on-going to determine their baseline growth factor dependence.

Baseline clonogenic survival assays were performed on high-risk cell lines 6-16 (HPV), 5-24, 7-31, and 11-21. The 3 control cell lines (11-6, 1-24, and 1-30) were also compared. Single doses of 2, 4, 6, 8, 10, and 15 Gy were used. Cell survival curves were superimposable for low- and high-risk curves. Examples of the curves are shown in Figures 5 and 6. For all cell lines irradiated, the dose of radiation resulting in approximately 10% cell kill was 5 Gy. This will be the radiotherapy dose used for future experiments.

#### Detailed Methods

Preparation of normal mammary epithelial cells: Breast tissues are minced with scalpels and incubated overnight in Medium 199 containing type III collagenase at a concentration of 200 units/ml and Dispase at 1mg/ml. Twenty ml of media/gram of tissue are used. The tissues are agitated gently in a shaking water bath at 37°C. The cells are then washed extensively in Medium 199, and an aliquot of the cells is counted by isolating and counting nuclei with a Coulter counter.

Excess cells from patient specimens not used in primary culture experiments are cryopreserved for future use. To freeze cells, they are suspended in Medium 199 supplemented with 20% FBS and 5% dimethylsulfoxide at a concentration of  $5 \times 10^6$  cells /750 ul of freezing medium. The cells are cooled slowly to -80°C using a step freezer and then stored over liquid nitrogen.

Cell culture: Mammary epithelial cells isolated by the methods described above are seeded onto collagen-coated (Vitrogen100, Collagen Corp.) 60-mm or 35-mm culture dishes at densities ranging from  $10^5$  to  $5 \times 10^5$  cells/dish. The complete serum-containing medium consists of Ham's F-12 (Hazelton Biologicals) supplemented with insulin (5 ug/ml), hydrocortisone (1ug/ml), epidermal growth factor (10ng/ml), cholera toxin (100 ng/ml), 5% fetal bovine serum, Gentamycin, and Fungizone.

To characterize specific growth factor requirements for proliferation of irradiated cells, a defined serum-free media will be used which contains Ham's F-12 supplemented with bovine serum albumin (1mg/ml), sodium selenite (50ng/ml), triiodothyronine (50ng/ml), Gentamycin, Fungizone with or without insulin, hydrocortisone, epidermal growth factor, and cholera toxin in the same concentrations as noted above.

Cells are maintained at  $37^\circ$  in a humidified atmosphere of 90% air and 10%  $CO_2$ . For subculture of cells grown in primary culture, cells are rinsed with calcium and magnesium-free Hanks' balanced salt solution and incubated with trypsin-EDTA for 2-5 minutes. The cells are reseeded at split ratios of 1:3 to 1:10.

Immortalization with E6 and E7 from human papilloma 16 virus: PA317 amphotrophic packaging cell lines stably transfected with pLXSN (vector), pLXSN16E6 (HPV-16 E6), or pLXSN16E7 (HPV-16 E7) with a G418 selectable marker (provided by V. Band, Tufts University School of Medicine) were grown to 70-80% confluence, and supernatants are collected for 16 hours and stored in aliquots at  $-80^\circ C$ . Approximately  $10^5$  cells are plated for 25- $cm^2$  flask for 18 hours and infected with 100-200ul of virus stock in 2 ml of medium containing Polybrese (Sigma) at 4ug/ml for 8 hours.

Differential cell isolation: Filter-selection methods are used to isolate individual colonies from primary cultures derived from normal or neoplastic tissues. Cells are seeded at low density ( $< 10^5$  cells per 60 mm dish), grown for 10 days, and then chosen for filter selection. For filter selection, small pieces of Whatman paper are soaked in trypsin-EDTA and then placed on individual colonies and incubated. Filters are then removed and placed in a 35-mm wells containing fresh growth medium. The plates are incubated overnight during which the cells detach from the filter and attach to the substratum. Cells are then grown to confluence and subcultured.

Exponential cell growth: Doubling times for each of the cells will be determined as follows:  $1 \times 10^4$  cells will be seeded in 35-mm 6-well plates. Cell nuclei will be enumerated using the Coulter counter at 2,6,10,14 and 18 days. All measurements will be made in triplicate.

Radiation cell survival: Irradiations will be performed at room temperature at 1.5 Gy/min using a Cobalt-6 source (AECL Theratron 80). Dosimetry calibration is carried out using an ionization chamber (Baldwin Farmer) connected to an electrometer system that is directly traceable to a National Bureau of Standards calibration. After radiation treatment, cells are assessed for clonogenic survival: Cells will be removed from the dishes with phosphate buffered saline containing trypsin-EDTA and diluted into culture dishes in numbers to yield between 20 and 200 colonies per plate. After 10-14 days, the plates were fixed with methanol-acetic acid, stained with crystal violet, and scored for colonies containing more than 50 cells. Radiation survival data will be corrected for plating efficiency using an unirradiated plate of cells. Cell survival curves will be fit using the linear-quadratic equation (4). Radiation sensitivity is expressed in terms of the mean inactivation dose, which represents the area under the cell survival curve. The mean inactivation dose will be calculated by linearizing the linear quadratic equation through use of a logarithmic transform, fitting alpha and beta by linear regression, and integrating over this curve using the method described by Fertil et al (5). Establishment of these curves will then allow us to select a dose of radiation resulting in 10 to 50% cell survival.

## **Conclusions**

The work done thus far has largely focused on characterizing the high-risk cell lines. Specifically, SSCP testing has been done to locate a BRCA-1 mutation that is a known functional mutation. While this testing is on-going, we continue to do the baseline experiments needed to complete the proposed work. All baseline radiation experiments have been done, showing that clonogenic survival is equivalent for the low-risk (control) and high-risk cell lines and that the dose resulting in approximately 10% survival in the HPV-infected cell lines is 5 Gy. Growth factor experiments done thus far have shown an EGF dependence in E6 E7 infected experiments. Other testing for growth factor independence is on-going, as are soft-agar assays and karyotyping. Although much work was done to characterize doubling times for the mammary epithelial cell lines, progression to senescence has prevented usage of these uninfected cells. Therefore, immortalization cannot be used as a differentiating phenotype for malignant progression between the control and high-risk cell lines. Instead, our endpoints will be growth factor independence, soft agar

growth, differences in FISH and karyotyping, and *in vivo* tumor growth in nude mice.

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## **APPENDIX**

# SSCP

Exon 11 I<sub>2</sub>

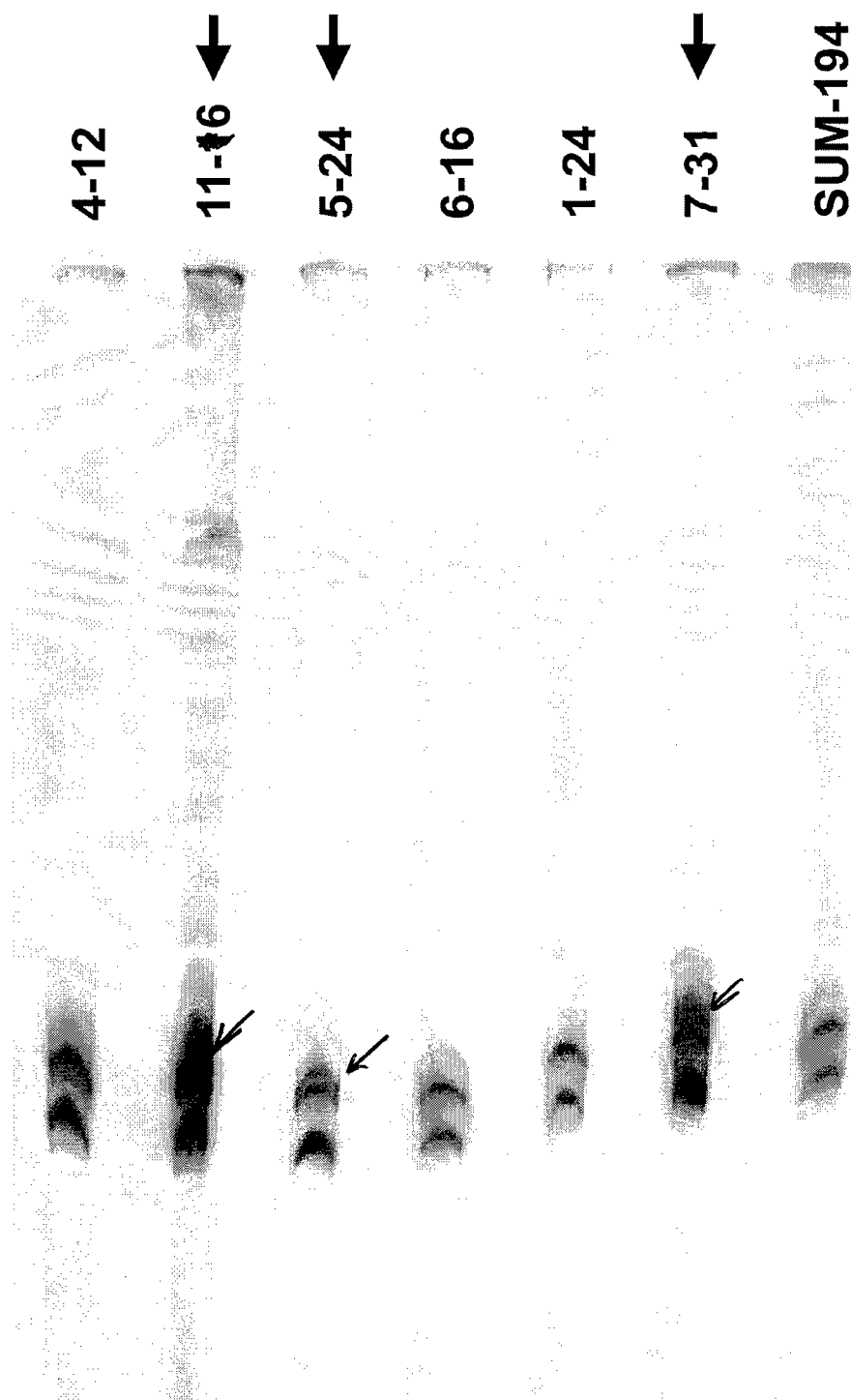


Figure 1

# CSGE

## Exon 11 I<sub>2</sub>

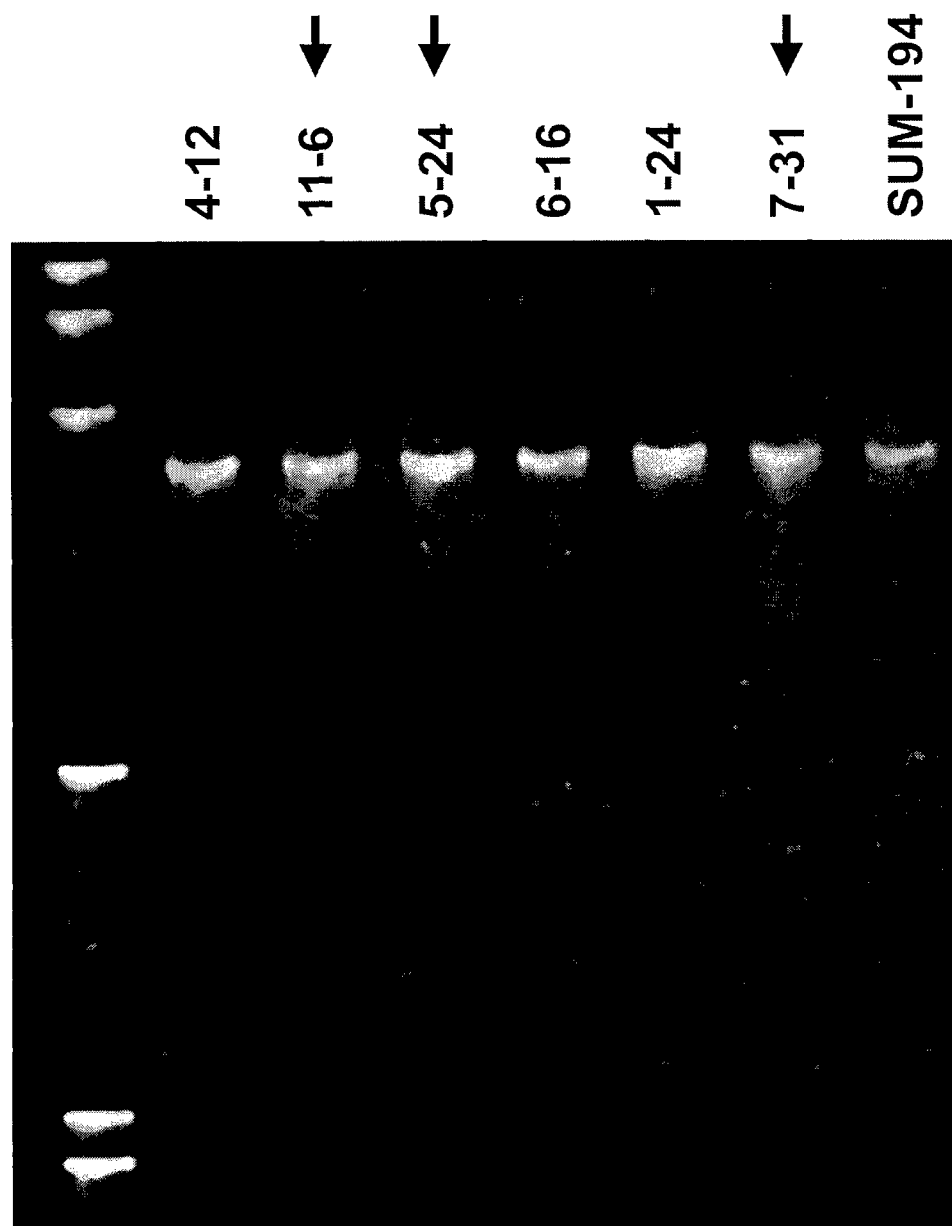


Figure 2

### HPV Cell Growth Experiment in SFIHC

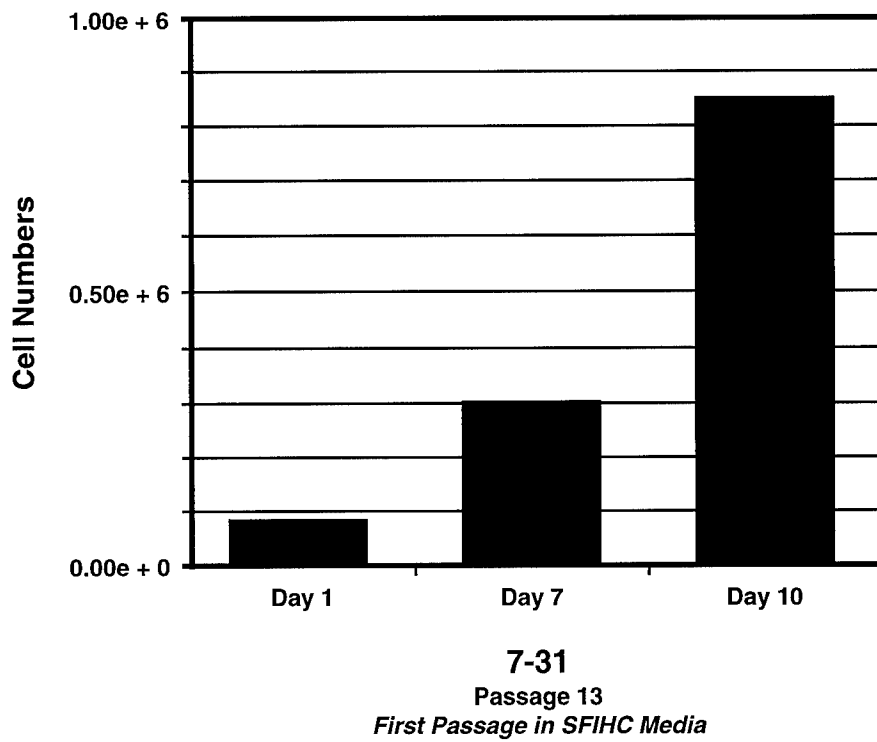


Figure 3

### HPV Cell Growth Experiment in SFIHC

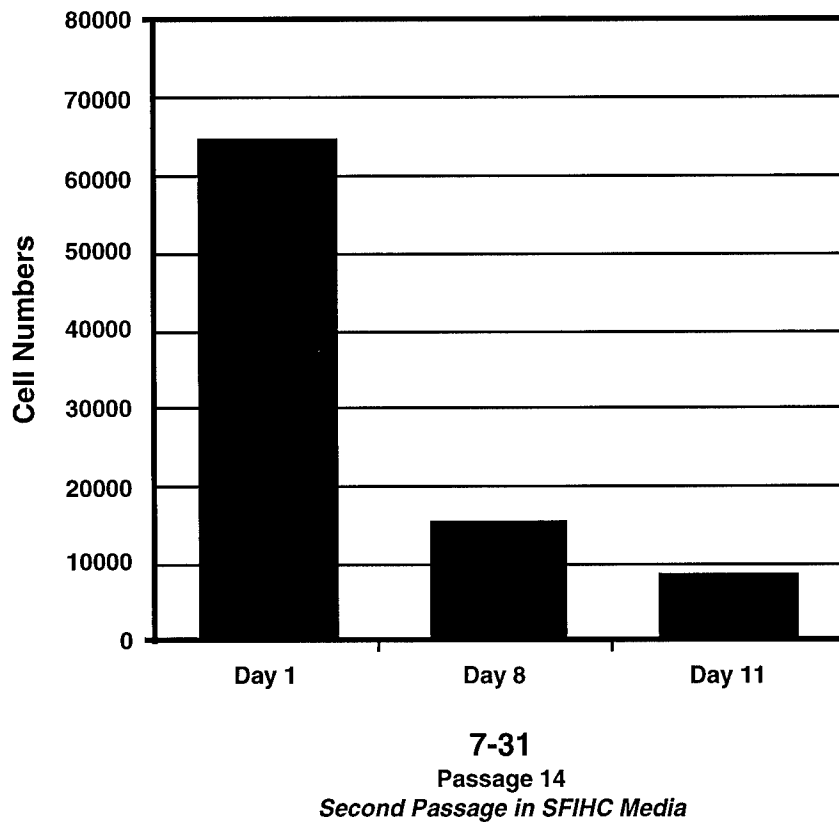


Figure 4

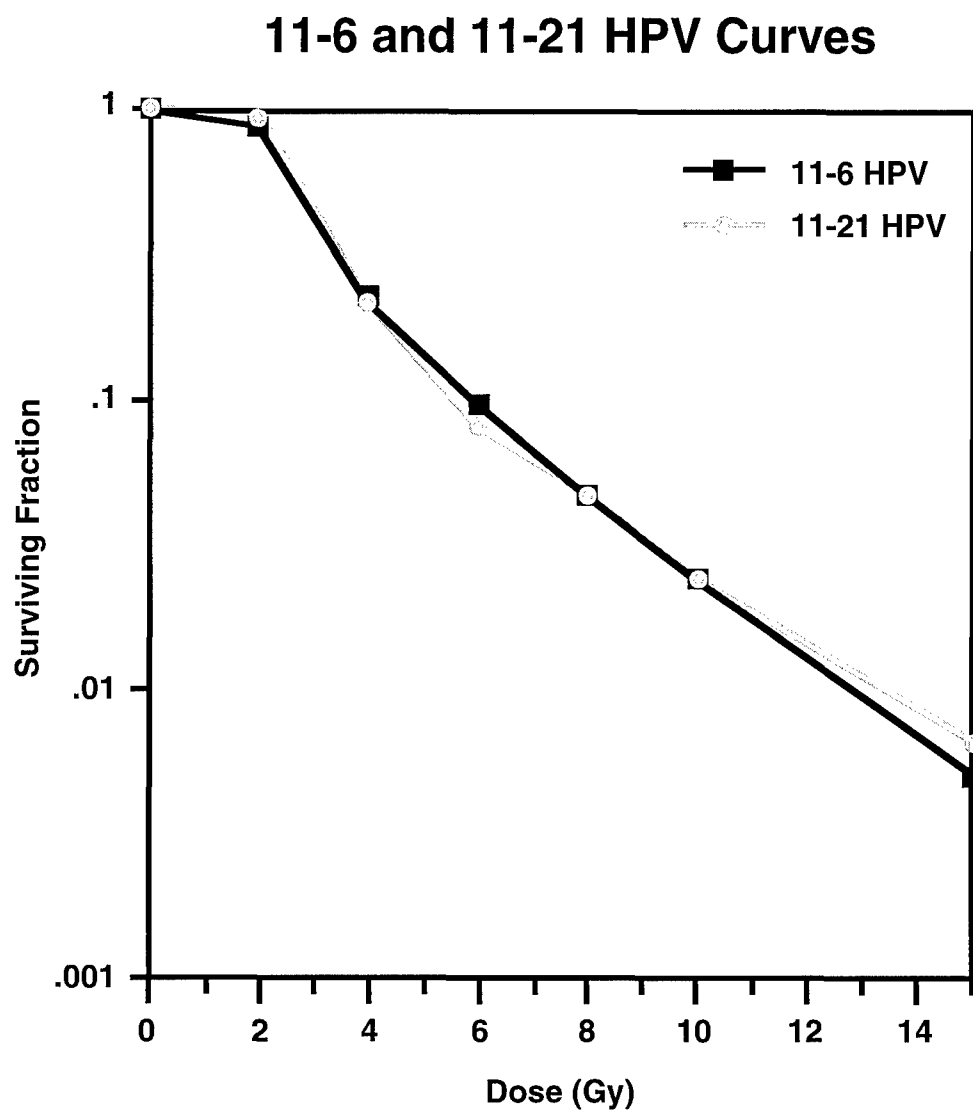


Figure 5

## 11-6, 1-24 and 1-30 HPV Curves

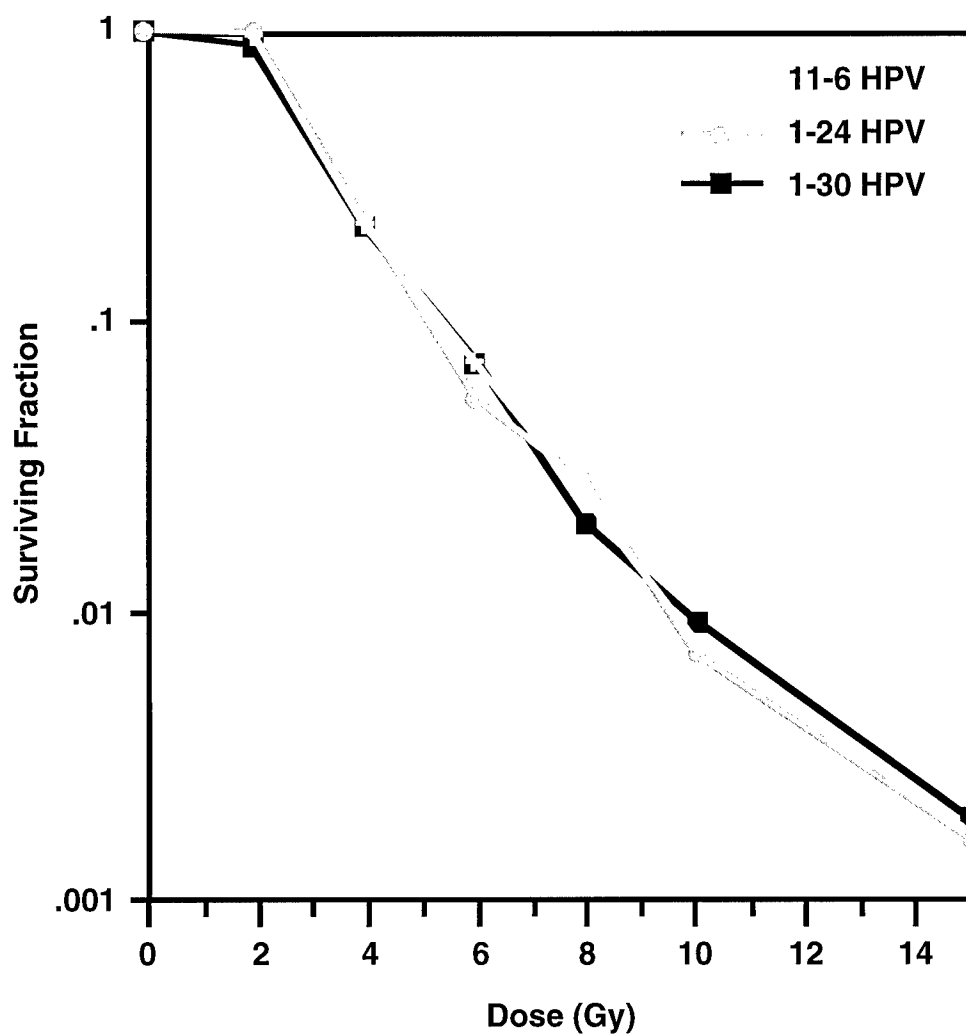


Figure 6